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# Apatite-forming ability of polyglutamic acid hydrogels in a body-simulating environment

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**Abstract** Artificial joints can replace damaged joints provided the surrounding bone is sufficiently dense. However, elderly patients generally have reduced osteoporosis-associated bone density. Therefore, restitution of bone density is essential to ensure implantation. Injectable and resorbable bioactive fillers with bone-bonding ability (osteoconductivity) are promising, as osteoporosis can be reversed with minimal invasion. Osteoconduction occurs through the surface formation of biologically active hydroxyapatite via reactions with body fluids. Heterogeneous nucleation of the hydroxyapatite is catalysed by specific surface functional groups. In addition, release of $Ca^{2+}$ ions into the surrounding fluids enhances apatite nucleation by increasing its degree of supersaturation. We tested injectable bioactive filler made from cross-linked polyglutamic acid (PGA). This has many carboxyl groups that facilitate apatite nucleation. An insoluble hydrogel can be formed by cross-linkage. We exposed PGA gels to a simulated body fluid for 7 days. Trace amounts of calcium phosphate were formed, but were not identified as bone-like apatite by X-ray diffraction. However, formation of a bone-like apatite layer was detected using pre-treatment with $CaCl_2$ solutions (>0.01 mol $dm^{-3}$) dose dependently. Thus, this chemically cross-linked PGA gel could induce the heterogeneous nucleation of hydroxyapatite in a body environment, and this was enhanced by pre-treatment with $CaCl_2$.

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## 1 Introduction

Prostheses such as artificial hip and knee joints are now commonly used to replace damaged joints. To bond these prostheses with the surrounding bone, two types of fixation have been adopted. One is fixation using self-setting polymethylmethacrylate (PMMA)-based bone cements to fill the gap between the artificial joints and the bone [1]. The other is the use of cement-free artificial joints [2]. These prostheses are fixed by anchoring the macroporous structure on their surfaces with the ingrown bone. Some of the prostheses are modified with a bioactive surface layer, because bioactive materials show osteoconductive properties—direct bonding to living bone—to provide tight fixation for long periods. Cement-free fixation of the artificial joints requires formation of regenerative bone tissue around the materials to lead to ingrowth of newly formed bone. In addition, normal cortical bone should be maintained around the implant to support the mechanical fixation. Nevertheless, elderly patients sometimes show poor osteogenesis. The bone density of such patients is generally lower than that of healthy people because of diseases such as osteoporosis. These problems make it difficult to fix the artificial joints to the bone tightly. Therefore, we have to pay attention to the restitution of normal bone density as an important parameter for the successful implantation of artificial joints.

This study focused on an osteoconductive, injectable bone filler based on a bioresorbable polymer as a potential treatment to increase bone density, because such injectable fillers are a minimally invasive means to help patients recover bone density damaged by osteoporosis. Bioresorbability is also required to avoid the tendency of residual materials to inhibit bone ingrowth on the surface of the artificial joint.

Previous studies have reported that bioactive materials show osteoconduction—direct bonding to living bone—by the formation of a surface layer of biologically active hydroxyapatite ("bone-like apatite") on their surfaces via chemical reactions with the surrounding body fluid [3, 4]. The formation of a bone-like apatite layer is initiated by heterogeneous nucleation on the surface of the materials caused by specific functional groups, such as SiOH [5, 6], TiOH [6, 7], ZrOH [8], TaOH [9], NbOH [10], carboxyl (COOH) groups and phosphate groups [11]. In addition, the release of $Ca^{2+}$ ions into the surrounding body fluids enhances apatite nucleation by increasing the degree of supersaturation with respect to the hydroxyapatite [12].

In the present study, we tested the ability of $\gamma$-polyglutamic acid (PGA) to develop bioactive materials with osteoconduction and bioresorbability. PGA is a polypeptide in which D- and L-glutamic acids are copolymerized to give the chemical structure shown in Fig. 1. PGA is a component of *Natto*, a traditional Japanese food produced from soybeans [13, 14]. PGA is the bioresorbable polymer that degrades into its component amino acids after implantation. We hypothesized that PGA might act as an osteoconductive biomaterial with high apatite-forming ability in the body because it is rich in carboxyl groups. Moreover, glutamic acid-rich sequences in osteonectin, a kind of non-collagenous protein found in bone and dentine, act as binding sites for hydroxyapatite crystals [15]. This suggests that PGA might have suitable properties to produce injectable bioactive materials with osteoconduction and bioresorbability.

PGA polymer is generally water-soluble and to be converted into an injectable gel form it should be cross-linked through an appropriate procedure. In the present study, we prepared PGA hydrogels through covalent cross-linking, using diamine and water-soluble carbodiimide to suppress rapid dissolution in aqueous environment. However, it was unclear whether the cross-linked PGA gel would show osteoconductivity in terms of bone-like apatite formation in a body environment. Osteoconductivity of the PGA gels was evaluated in vitro from the apatite-forming ability of the gels after exposure to a simulated body fluid (SBF) proposed by Kokubo et al., which can support the formation of bioactive materials with osteoconduction [16–18]. The feasibility of enhancement by prior treatment of the PGA gels with aqueous $CaCl_2$ was also investigated.

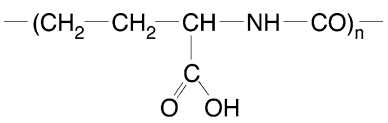


**Fig. 1** Chemical structure of polyglutamic acid (PGA)

## 2 Materials and methods

We used PGA produced by *Bacillus* strain (Meiji Food Materia Co., Ltd., Tokyo, Japan), which is partially cross-linked by gamma irradiation, but is easily dissolved in aqueous media. Covalent cross-linking was performed as described [19, 20]. The PGA powder was dissolved in distilled water to form aqueous solutions with concentrations of 5% (w/w). Ethylenediamine-2(*N*-hydroxysuccinimide) was prepared by adding 150 $cm^3$ of 1.7% 2*N*-hydroxysuccinimide ethyl acetate solution drop-wise to 10 $cm^3$ of 6.3% ethylenediamine ethyl acetate solution. Then, 0.2 g of ethylenediamine-2(*N*-hydroxysuccinimide) and 0.96 g of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC · HCl) were added to 30 $cm^3$ of the PGA solution to progress the cross-linking reaction. The solution was poured into a Teflon-coated dish and kept at room temperature for 24 h. The gels obtained were cut into rectangular pieces measuring 10 × 10 × 1 mm and treated with 30 $cm^3$ of $CaCl_2$ aqueous solutions at various concentrations ranging from 0.01 M to 1 M (= mol $m^{-3}$) at 36.5 °C for 24 h, followed by rinsing with ultrapure water.

The untreated and $CaCl_2$-treated gels were then soaked in 30 $cm^3$ of SBF ($Na^+$ 142.0, $K^+$ 5.0, $Mg^{2+}$ 1.5, $Ca^{2+}$ 2.5, $Cl^-$ 147.8, $HCO_3^-$ 4.2, $HPO_4^{2-}$ 1.0 and $SO_4^{2-}$ 0.5 in mol $m^{-3}$). SBF was prepared by dissolving reagent-grade chemicals of NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2$ and $Na_2SO_4$ in ultrapure water and buffering at pH 7.40 with tris(hydroxymethyl)aminomethane ($(CH_2OH)_3CNH_2$) and an appropriate volume of hydrochloric acid (HCl), as described in the literature [17]. All the reagents used to prepare SBF were supplied by Nacalai Tesque, Inc., Kyoto, Japan. The solution was kept at 36.5 °C for various periods of up to 7 days. After soaking in SBF, the specimens were removed from the fluid, washed gently with ultrapure water and dried at room temperature.

The surface structural features of the specimens before and after soaking in SBF were examined by scanning electron microscopy (SEM; S-3500N, Hitachi Ltd., Tokyo, Japan) using energy-dispersive X-ray microanalyzer (EDX; EMAX Energy, Horiba Ltd., Kyoto, Japan), Fourier-transform infrared spectroscopy (FT-IR; Spectrum GX2000R,

PerkinElmer Inc., MA, USA) and thin-film X-ray diffraction (TF-XRD; MXP3V, MAC Science Ltd., Yokohama, Japan). For FT-IR, the gels were first pulverized and mixed with KBr powder at a mass ratio of 1:100. A thin film was prepared by pressing the mixed powder, which was then measured. For TF-XRD, the angle of the incident beam was fixed at 1° against the surface of the specimen and the measurements were performed using a step scanning mode with steps at 0.02° steps and 1 s. For SEM observation, a thin film of gold was sputter-coated on the surfaces of the specimens.

## 3 Results

Figure 2 shows SEM micrographs of the surfaces of PGA gels with and without $CaCl_2$ pre-treatment, after soaking in SBF for 7 days. Spherical particles about 2–3 μm in diameter were observed for the treated gels, irrespective of the concentration of the $CaCl_2$ used. Figure 3 shows TF-XRD patterns of the PGA gels with and without $CaCl_2$ pre-treatment. Tiny broad peaks at 26° and 32° in $2\theta$ were detected in the diffraction patterns of the PGA gels treated with $CaCl_2$ solutions irrespective of their concentrations. The peak at about 32° in $2\theta$ was assigned to a diffraction envelope of (211), (112), (300) that resulted from low-crystalline hydroxyapatite (JCPDS Card # 15-0876), while that at 26° in $2\theta$ was assigned to (002) diffraction. On the other hand, these peaks were not detected from the untreated PGA gels. Figure 4 shows EDX spectra of the surfaces of PGA gels with and without $CaCl_2$ pre-treatment. Peaks assigned to Ca and P were detected for all the gels. The small peak assigned to P was detected even in the sample not treated with $CaCl_2$. The intensity of the peaks increased with increasing concentration of $CaCl_2$ solution. The increases in both peaks represented increases in calcium phosphate forms, including hydroxyapatite. Figure 5 shows SEM micrographs and EDX spectra of the cross-sections of

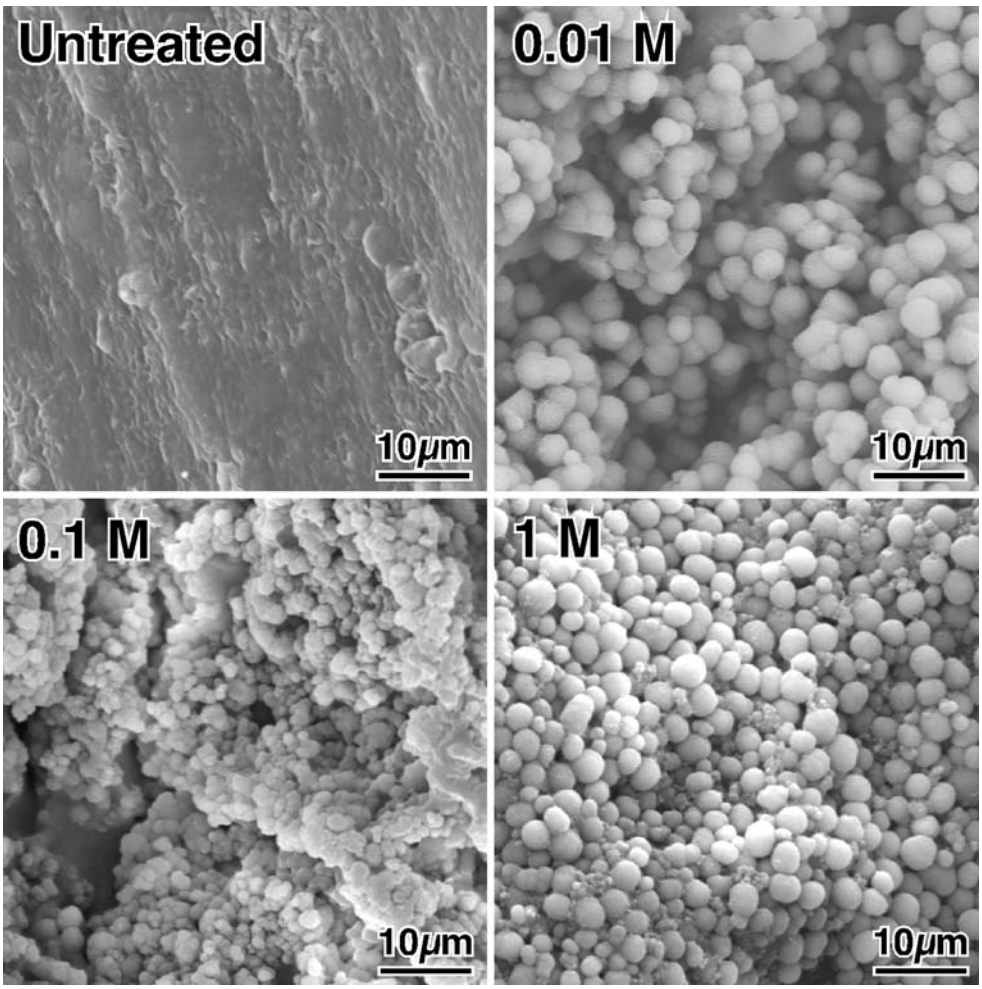


**Fig. 2** SEM photographs of the surfaces of PGA gels with and without $CaCl_2$ treatment, after soaking in SBF for 7 days

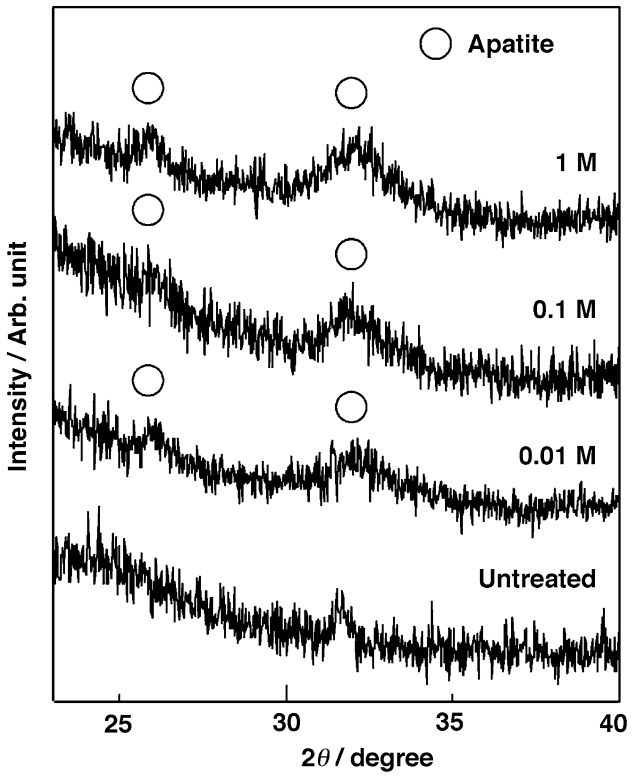


**Fig. 3** TF-XRD patterns of the PGA gels with and without $CaCl_2$ treatment, after soaking in SBF for 7 days

PGA gels pre-treated with 1 M $CaCl_2$ solution. Three points where EDX analysis was performed are indicated. Note that Ca and P peaks were detected not only on the top surface of the gel but also in a region about 40 μm deep. The intensity of the Ca and P peaks decreased away from the surface of the gel. Thus, calcium phosphates were present, implying hydroxyapatite could form inside the gel structure. Figure 6 shows FT-IR spectra of the PGA gels treated with or without 1 M $CaCl_2$ solution, before and after soaking in SBF for 7 days. Peaks assigned to carboxyl groups were detected on both PGA gels before soaking in SBF at about 1,400 and 1,600 $cm^{-1}$. After soaking in SBF for 7 days, the peaks assigned to P–O bending and P–O stretching were detected for both the gels at about 600 and 1,000 $cm^{-1}$, respectively. In addition, the gels treated with $CaCl_2$ gave splitting of the peaks at 1,050 and 1,120 $cm^{-1}$ in combination with the appearance of two peaks at about 560 and 620 $cm^{-1}$. This feature is characteristic of crystalline apatite [21]. Thus, the PGA gel without $CaCl_2$ treatment could form amorphous calcium phosphate.

Table 1 summarizes apatite formation in the examined PGA gels soaked in SBF for various periods. Apatite formation was determined by detection of the peaks at 26° and 32° in $2\theta$ on the TF-XRD patterns. Clearly, the time taken to induce apatite formation decreased with increasing $CaCl_2$ concentration.

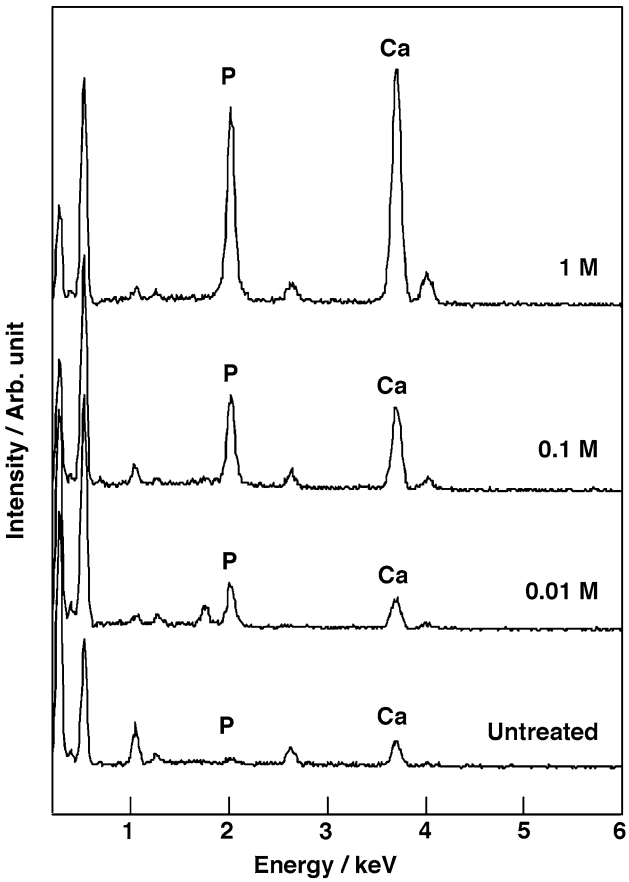


**Fig. 4** EDX spectra of the surfaces of PGA gels with and without $CaCl_2$ treatment, after soaking in SBF for 7 days

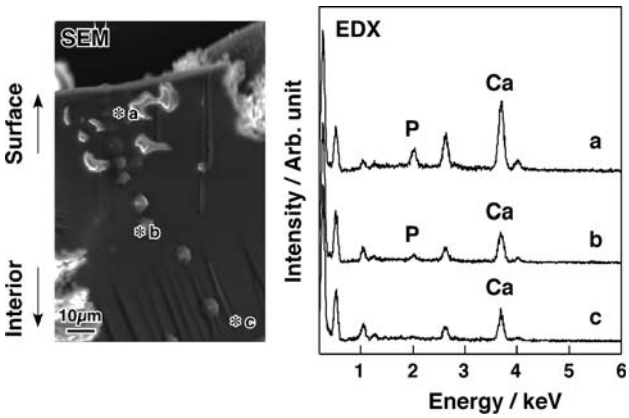


**Fig. 5** SEM photographs and EDX spectra of the cross-sections of PGA gels treated with 1 M-$CaCl_2$ solution, after soaking in SBF for 7 days

## 4 Discussion

It is evident from our results that chemically cross-linked PGA gels have the potential to deposit hydroxyapatite in a body-simulating environment when treated with $CaCl_2$ aqueous solution at concentrations of 0.01 mol $dm^{-3}$ or

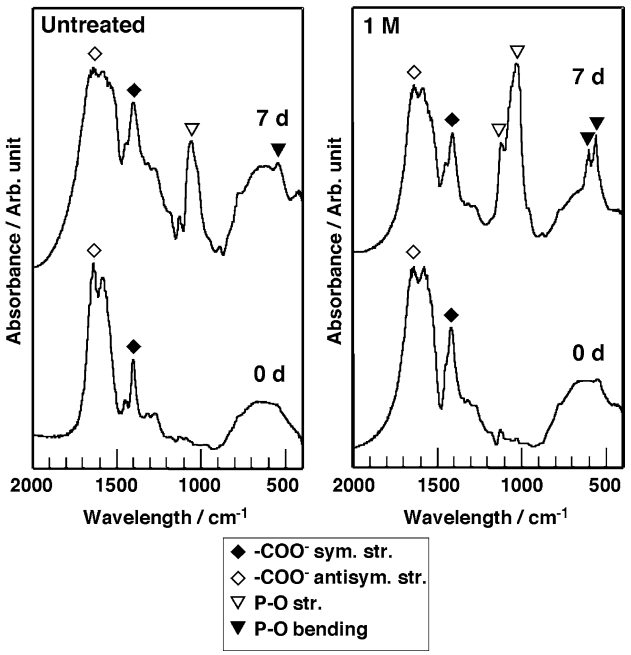


**Fig. 6** FT-IR spectra of the PGA gels modified with or without 1M-$CaCl_2$ solution before and after soaking in SBF for 7 days

**Table 1** Apatite formation on PGA gels with or without $CaCl_2$ treatment in SBF, which was determined by detection with TF-XRD

| $CaCl_2$ concentration | Soaking time in SBF | | |
|---|---|---|---|
| | 1 day | 3 days | 7 days |
| Untreated | − | − | − |
| 0.01 M | − | + | + |
| 0.1 M | − | + | + |
| 1 M | + | + | + |

+: Apatite was detected

−: Apatite was not detected

greater. Thus, these PGA gels treated with $CaCl_2$ have a high potential to exhibit osteoconduction after implantation in bony defects. The PGA gels achieve this form after soaking in SBF. It is interesting that even the untreated PGA gels formed amorphous calcium phosphate after soaking in SBF for 7 days. This suggests that PGA gels themselves can calcify in body environments. Takadama et al. investigated the precise mechanism of apatite formation on various bioactive materials, such as calcium silicate-based glasses and NaOH-treated titanium metals in SBF, using transmission electron microscopy (TEM) observation and X-ray photoelectron spectroscopy (XPS) [22–25]. They showed that apatite nucleation progressed on the surfaces of these materials through formation of an amorphous calcium phosphate and subsequent conversion into the apatite. The formation of calcium phosphate on the PGA gels without $CaCl_2$ treatment after soaking in SBF indicates that the carboxyl groups on and in the gel structure may act as a site to make heterogeneous nucleation of amorphous calcium phosphate. Subsequent crystallization of the apatite did not occur when the PGA gels were not treated with $CaCl_2$. Incorporation of $Ca^{2+}$ ions near or on the carboxyl groups in the gels helps to increase the degree of hydroxyapatite supersaturation and thus allows further crystallization. Thus, we assume that the calcium phosphate formed in the untreated PGA gels may be merely a precursor state of the apatite and too stable to convert into the apatite in SBF. However, apatite formation in the PGA gels treated with $CaCl_2$ solution was accelerated by increases in concentration of $CaCl_2$. Thus, coexistence of $Ca^{2+}$ ions near carboxyl groups can increase the rate of the apatite formation in PGA gels soaked in SBF.

The chemically cross-linked gel was strong enough to be used for the TF-XRD measurements, but could also withstand injection with an 18-G needle. Injectability, strength and swelling properties could be controlled by process of cross-linkage. Interestingly, the swelling properties permitted the formation of apatite inside the gel structure, as shown by EDX analysis (Fig. 5). PGA is an organic polymer with high swelling properties [13]. Apatite deposition inside the gels was induced around the penetrated fluid. Such an environment would be favourable for apatite deposition, because local increases in $Ca^{2+}$ concentration in the surrounding fluid can be maintained. For the application of apatite-polymer porous materials as scaffolds for bone regeneration, the formation of a bioactive layer inside the pores is desirable for achieving healthy bone ingrowth [26]. Apatite can be formed on the surfaces of synthetic aromatic polyamide films [27] and silk sericin [28], which contain carboxyl groups when they are pre-treated with $CaCl_2$. These polymers formed the apatite on their surfaces only in modified SBF (1.5 SBF) with inorganic ion concentrations 1.5 times that used here. The proportions of carboxyl groups contained in the synthetic aromatic polyamide, silk sericin and PGA were calculated to be 8.7, 9.6 and 34.9% (w/w), respectively. The high apatite-forming ability of PGA is attributed to the high concentration of carboxyl groups. Based on these findings, PGA appears to be a strong candidate for the formation of porous scaffold materials.

## 5 Conclusions

Hydrogels of PGA were prepared by chemical covalent cross-linking using diamine. The PGA gels formed large amounts of apatite in SBF, when first treated with aqueous $CaCl_2$ solutions. The apatite-forming ability was enhanced with increases in $CaCl_2$ concentration. This type of PGA

hydrogel is expected to be used as injectable bone filler with bioactivity and bioresorbability for bone tissue reconstruction.

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